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STUDIES ON THE PENETRATION OF THE BLOOD-BRAIN
BARRIER BY CANCER CHEMOTHERAPEUTIC AGENTS

MALIN ROY DOLLINGER

1960

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Dear Dr. Forbes:

This thesis of Malin Roy Dollinger has been
read and approved by me as acceptable for
presentation as partial fulfillment of the
requirements for the degree of Doctor of Medicine.

Sincerely yours,



Arnold D. Welch, M.D.
Chairman

ADW:bjh



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STUDIES ON THE PENETRATION OF THE BLOOD-BRAIN
BARRIER BY CANCER CHEMOTHERAPEUTIC AGENTS

By

Malin Roy Dollinger, A.B.
Stanford University, 1956

A thesis submitted to the faculty of the Yale
University School of Medicine in partial ful-
fillment of the requirements for the degree of
Doctor of Medicine

Department of Pharmacology

1960



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I. INTRODUCTION

A. The blood-brain barrier: anatomic, physiologic, and pharmacologic concepts.

The brain appears to be in a class by itself insofar as the penetration of compounds is concerned. It has been observed that there is a selective penetration of substances from the blood into the cerebrospinal fluid in mammals. Normal constituents of the blood may be almost totally excluded from the cerebrospinal fluid or they may appear in concentrations similar to those in plasma(1). Alterations in the plasma concentrations of oxygen, glucose, urea, sodium ions, and chloride ions, for example, are followed by comparable alterations in the concentrations of these substances in the cerebrospinal fluid. No substances are found in the cerebrospinal fluid which are not normally found in the blood(2). Mesobilirubin, on the other hand, does not penetrate into the brain of the adult, although there is apparently no barrier to the entrance of this substance into the brain of the newborn infant(3). This forms the basis for the development of kernicterus(pigmentation of the basal ganglia of the brain by bilirubin) in infants in whom the serum indirect-reacting bilirubin level exceeds 20 mg/100 ml, as in erythroblastosis fetalis. Adults with levels of serum bilirubin high enough to produce clinical jaundice never develop kernicterus, although the serum bilirubin level may exceed the above value.

Similarly, drugs introduced into the blood may penetrate into the brain without difficulty or may pass into the central nervous system slowly or not at all, even though penetrating other organs with ease.

The boundary between the brain and the blood is generally called the blood-brain barrier, or hemoencephalic barrier(4-8). It should be noted that this barrier does not lie between brain cells and the fluid surrounding them, since drugs are

known to penetrate the cells of the brain as readily as those of any other tissue(9). The barrier seems to lie between plasma and the extracellular fluid of the central nervous system(10). It will be assumed here that the cerebrospinal fluid and the extracellular fluid of the central nervous system constitute a continuum, and that measurements made on the former reflect accurately changes occurring in the latter(11). Mayer has furnished evidence which supports this concept(12). Measurements of the influx of drugs into the central nervous system at different stages of equilibrium indicate that the blood-cerebrospinal fluid and blood-brain barriers are essentially the same with respect to foreign compounds. Substances such as barbital, acetanilid, and N-acetyl-4-aminoantipyrine, which do not appreciably bind onto cellular components, enter the cerebrospinal fluid at the same rate as into brain water; although they penetrate at a very slow rate into the central nervous system. With thiopental, however, the concentration in brain water is much higher than in cerebrospinal fluid. The high brain/cerebrospinal fluid ratio observed here is not due to a high concentration gradient of drug between brain and cerebrospinal fluid but to reversible binding of the drug onto tissue components in the brain. Broman has also furnished considerable evidence substantiating the hypothesis that the cerebrospinal fluid and the extracellular fluid of the central nervous system reflect a continuum(10). The blood-brain barrier concept is supported by anatomic, physiologic, and pharmacologic evidence.

The first consideration is that of the location of the barrier. In experiments described by Brodie and Hogben the highly lipid-soluble drug amidopyrine penetrated into the brain so rapidly that it must have entered from blood capillaries in all parts of the brain(10). The concentration of this drug was uniform in various parts of the brain at any given time from two minutes to one hour after administration. The concept of uniformity of the barrier throughout the brain

is also substantiated by the even distribution of N-acetyl-4-aminoantipyrine in cerebrospinal fluid, cerebral cortex, cerebellum, brain stem, and medulla shortly after drug administration(12). From these results it may be inferred that the flow of drugs into cerebrospinal fluid is primarily by way of the choroid plexus and perhaps from pial vessels, whereas drugs pass into brain tissue directly from brain capillaries(13). A similar conclusion as to the uniformity of the blood-brain barrier to bromide, iodide, and thiocyanate ions was reached by Wallace and Brodie(14). It must be emphasized, however, that for substances such as glucose and sodium which enter the central nervous system by specialized transfer mechanisms, the rates of influx may well vary in different parts of the central nervous system.

Blood-cerebrospinal fluid partitions seem to be more complex structurally than those separating blood vessels from extracellular fluid elsewhere in the body. Capillaries in the choroid plexuses of the brain, from which the cerebrospinal fluid is evolved, are separated from the cerebrospinal fluid by a layer of ependymal cells. Elsewhere in the central nervous system the endothelium of capillaries appears to be covered by a continuous sheath of neuroglial(astrocytic) cytoplasmic processes. Materials from the blood may thus be required to pass through the glial cells before they become available to the neurons(2,15). Perhaps these unique features of cerebrospinal blood vessels are related to the special physiological relations between the bloodstream on one hand, and the cerebrospinal fluid and interstitial fluid of the central nervous system on the other.

Most of the exchange of materials between the brain and the rest of the body occurs by this route, including both normal metabolites and administered substances. Exclusion appears to be exerted just beyond the cerebral capillaries. Microscopic observation of these capillaries after intravenous injection of colored and fluorescent substances indicates that they penetrate into the brain

as far as the cells of the capillaries, but not to the glial cells which surround them. The electron microscopic ultrastructure of this perivascular space has been elucidated only recently(16).

It is noteworthy that differential permeability is seen to appear in the brain at a stage in development when the lining of glial processes also appears-- and again, that certain limited areas of the brain, in which the glial cell lining is absent, do not show a barrier such as is found elsewhere in the brain. These areas, the hypothalamus, pituitary, and pineal, differ from the rest of the brain in that they are readily colored by intravenously administered vital dyes which do not penetrate other parts of the brain; and intravenously administered P³² appears in these locations much more rapidly than in other parts of the brain(17,18,19). The blood-brain barrier may be functionally different in these instances. It is interesting to note that all three of these regions have a neuroendocrine function.

There are several physiological lines of evidence favoring the existence of a blood-brain barrier. As mentioned previously, bile pigments do not penetrate into the brain of the adult regardless of their blood concentration; whereas the development of basal ganglia pigmentation(kernicterus) is presumably due to immaturity of this barrier during the first week of life.

The permeability of the barrier is occasionally altered in persons with brain tumors. This permits localization of such tumors with iodofluorescein, which does not penetrate the blood-brain barrier in normal individuals(20). Similarly, dyes containing radioactive iodine(I¹³¹) can be used to localize brain tumors, using appropriate detection devices(21).

Immunological techniques have shown that large molecules, such as proteins, are excluded from the brain(15). Glucose is one of the relatively few circulating nutrients capable of passing from the blood into the brain and of being effectively used by the cells of the central nervous system;

it appears to be actively transported(22). Radioactive ions introduced into the blood appear in the cerebrospinal fluid only after a considerable delay, indicating that the normal exchange of ions is exceedingly slow. For example, P³² levels in brain tissue reach a peak within one hour after direct introduction into the cerebrospinal fluid, whereas 12 to 24 hours are required after intravenous administration(23). Central nervous system tissue has a rapid phosphorous metabolism, but its access to vascular phosphate is normally severely limited(24). In pathological conditions such as inflammation or following brain laceration, phosphate is noted to enter the brain rapidly, in contradistinction to the above.

Sodium and chloride ions are not found in the cerebrospinal fluid in the quantities predicted by the Gibbs-Donnan theory of membrane equilibria. Radioactive sodium(Na²⁴) enters the brain slowly after intravenous administration, the "half equilibrium value" being 95± 6 minutes(25). This is contrasted with the time required for equilibration with the sodium in the rest of the body, which is measured in minutes rather than hours. These observations, together with the fact that administered radioactive sodium enters cells in the central nervous system slowly, give some insight into the nature of the blood-brain barrier(15,26). A similar effect is noted upon administration of radioactive chloride ion, in that penetration into the brain is relatively slow. This emphasizes that the "barrier" is specific to exchange with the blood(27).

Many other ionized substances penetrate only slowly into the brain from the blood. Ferricyanide given intraventricularly gives rise to marked central effects terminating in convulsions; this reaction does not occur after intravenous administration. Bromide, iodide, and thiocyanate ions also penetrate at a very slow rate.

Among several classes of substances, basic compounds usually penetrate into the brain more rapidly than acidic ones, although exceptions are known. In 1887 Ehrlich injected

acidic and basic aniline dyes into experimental animals; he observed that the basic substances, including methylene blue, reached the brain much as they did other organs of the body(28).

The brain in very young animals is less selective in this respect. The brain of the newborn mouse, for example, was stained by trypan blue, whereas the adult brain was not. The impermeability of the adult human brain to bilirubin illustrates the protective value of relative impermeability to acidic substances, suggested also in relation to many synthetic drugs of this nature.

Insulin does not pass the blood-brain barrier and is therefore excluded from the central nervous system. Since the central nervous system contains very little glycogen, it requires a continuous supply of available glucose in the extracellular fluid(20).

Glutamine penetrates the blood-brain barrier, although glutamic acid does not. Since the barrier seems to apply to exit of substances from the central nervous system as well as to entry, the glutamic acid formed within the brain cannot leave without being first combined with ammonia to form glutamine. This reaction is catalyzed by glutamine synthetase, an enzyme occurring in brain, retina, and kidney, and requiring magnesium ion and ATP as cofactors(20).

The trypanocide suramin, which is normally relatively non-toxic, becomes highly toxic when injected into the subarachnoid space, illustrating the teleological advantage of the barrier in excluding certain foreign substances. Binding of the drug to serum proteins also contributes to the difference in its effect when given by the two routes, in this particular case.

Calcium, magnesium, or potassium excess or depletion has a deleterious effect on vasomotor and respiratory reflexes. By means of the blood-brain barrier the central nervous system is protected against abrupt changes in its chemical

environment. Perhaps certain substances, such as potassium, are confined by this barrier to the central nervous system, where they are re-used by neurons and not swept away by blood(29). Tschirgi has suggested that the barrier serves to regulate the environment of the cells of the central nervous system(20,23). Homeostatic mechanisms may be even more highly developed in the brain than elsewhere in the body.

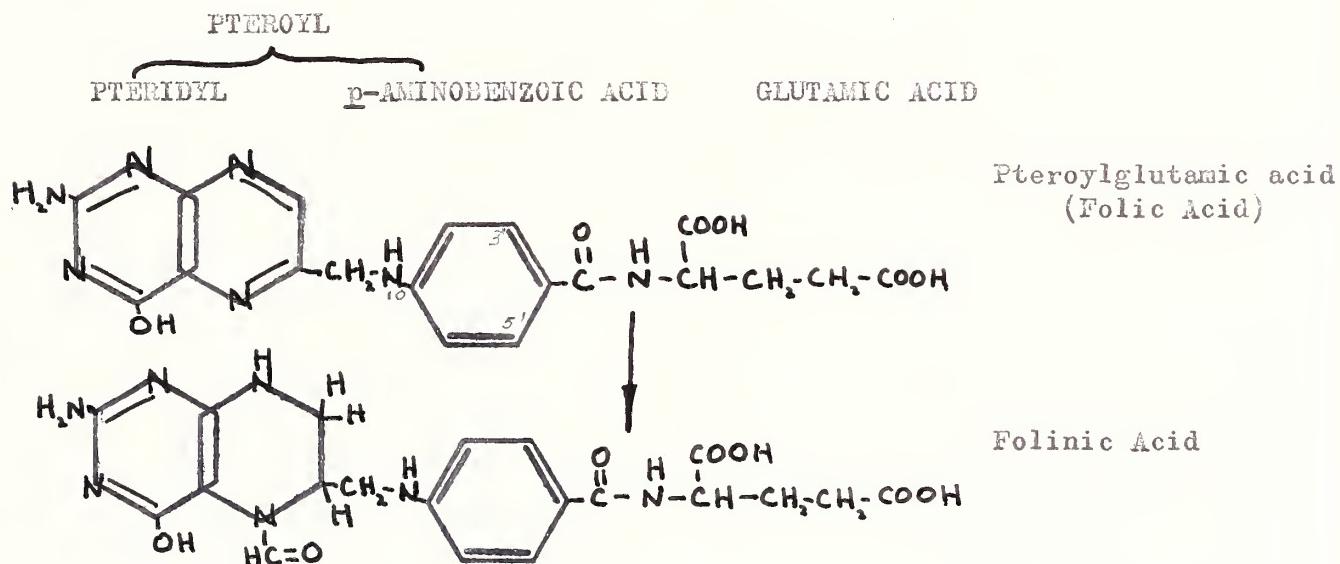
B. Folic acid and its antimetabolites; clinical uses of amethopterin.

Folic acid, or pteroylglutamic acid, was first implicated in human nutrition in 1931, when Wills described a macrocytic anemia in Hindu women in Bombay, usually associated with pregnancy, which responded to autolyzed yeast therapy(30). Wills and associates reproduced this anemia in monkeys by feeding them a diet similar to that of their human patients(31). It was also shown that this macrocytic anemia responded to crude liver extract, but not to the fraction effective in the treatment of pernicious anemia(32). The responsible fraction came to be known as the "Wills factor." Day et al in 1935 described an anemia in monkeys correctable by dried brewer's yeast, but not by any of the B complex vitamins known at that time(33). The unknown factor involved was called vitamin M. Stokstad and Manning in 1938, and Hogan and Parrott in 1940 described a syndrome in chicks characterized by anemia and growth failure; this was correctable by many substances, including yeast. The factor involved was labelled vitamin B_c(34,35).

Several microbiologists in the years 1940 to 1945 identified a substance necessary for the growth of Lactobacillus casei and called it the L. casei factor(36,37). Mitchell et al in 1941 isolated from spinach an acid which supported growth of both Strep. faecalis and L. casei; the leafy vegetable origin of this substance led to the name folic acid(38). During the next several years it was realized that the L. casei factor, vitamin M, and vitamin B_c were probably identical(39,40,41). Progress had been hampered

by the small yields of material available from natural sources, such as yeast or liver. The discovery by the Lederle Laboratories of an organism which synthesized the factor made available a sufficient quantity of the substance to enable Angier et al in 1946 to identify it chemically as a conjugate of pteroyleglutamic acid(42,43). Angier then synthesized the vitamin, and it was found that the hematological but not the neurological lesions (subacute combined degeneration) of Addisonian pernicious anemia could be alleviated by the vitamin(43).

Folic acid is apparently not active as such but is the precursor of the active vitamin, now known as folinic acid or citrovorum factor, since it is a growth requirement for Leuconostoc citrovorum(44). The biological conversion involves reduction of the folic acid molecule and introduction of a formyl group therein, and can be represented as follows:



This conversion is accomplished primarily in the liver and bone marrow(45,46). The action of the folic acid antagonists to be described later is to block this conversion and thereby to interfere with the important metabolic actions of the vitamin. The current belief is that tetrahydro folic acid

and its formyl derivative, folinic acid, act as prosthetic groups in enzyme systems involved in the formation and utilization of active one-carbon units, the so-called "formate" pool(47,48). In particular, folic acid has been shown to be important in methylation of the pyrimidine ring to produce thymine and in the introduction of the 2- and 8- carbon atoms into the purine ring. Its importance in nucleic acid metabolism is easily apparent.

Certain analogs of folic acid block the important actions of folic acid in nucleic acid metabolism and cytopoiesis. Studies by Franklin et al in 1947 showed that administration to animals of a crude methylated derivative of folic acid resulted in the signs and symptoms of folic acid deficiency, among which were slow growth, anemia, and leukopenia(49). Some degree of hematological remission was found when this antimetabolite was used in the treatment of leukemia. Interestingly, the administration of folic acid resulted in an exacerbation of the disease(50). Present pharmacologic interest centers around two active antimetabolites of folic acid: 4-aminopteroylglutamic acid(aminopterin), and 4-amino- N^{10} -methylpteroylglutamic acid(amethopterin, or Methotrexate®).

A great variety of analogs of pteroylglutamic acid have been synthesized and tested for possible growth inhibitory capacity against L. casei, as a screening method for possible use in man.

Cosulich et al prepared N^{10} -ethyl, butyl, carboxymethyl, benzyl, and phenacylpteroic acids, and showed these to be of a lower order of antagonist activity as compared to N^{10} -methylpteroylglutamic acid and N^{10} -methylpteroic acid(51).

The same group prepared 9-methyl and 9,10-dimethyl- pteroylglutamic acids and their 4-amino derivatives, and showed that they exhibited antagonism for pteroylglutamic acid in the growth of S. faecalis R.(52).

Seeger et al prepared several 4-amino derivatives of pteroylglutamic acid with growth antagonism activities of

from 1.6 to 15.3, as compared to 1.0 for half-maximum inhibition of growth of S. faecalis R.(53).

Wright et al replaced the glutamic acid moiety with several other amino acids and prepared the 4-amino derivatives of these substances. Bacterial growth inhibition was not significant, however(54).

Roth et al found that 4-(dimethylamino)-pteroylglutamic acid and its N¹⁰- derivative had a much lower antagonist activity for S. faecalis R. than does 4-aminopteroylglutamic acid. Also the toxicity was less for experimental animals(55).

Cosulich et al tested 3',5'-dihalopteroyl derivatives for activity against S. faecalis R. and found that 3',5'-dibromo-4-aminopteroylglutamic acid and 3',5'-dichloro-4-aminopteroylglutamic acid had slight growth antagonism activities as compared to amethopterin(56).

This same group has synthesized many other derivatives, including 2-alkylamino derivatives, 3'-chloro and 3'-bromo derivatives of amethopterin, and 4-alkoxy derivatives(57,58,59). In addition various tetrahydro compounds with a N¹⁰-formyl- pteroylglutamic acid nucieus corresponding to the above derivatives have been prepared, analogous to folinic acid(60,61,62).

Goldin et al showed that 3',5'-dichloroamethopterin has marked activity against L1210 leukemia in mice(63). N¹⁰-ethyl-amethopterin was shown to have the same antileukemic effectiveness as does amethopterin.

Collier and Phillips investigated 2,4-diaminopteridines as folic acid antagonists, and reported experiments with 6,7-dimethyl, diethyl, diphenyl, di-n-butyl, di-i-propyl, etc., derivatives, in which these compounds were tested for growth inhibition with Leuconostoc citrovorum cultures to which folinic acid had been added. The amount of growth was detected by titration of the acid produced in the medium. Peak activity was found with di-sec-butyl, di-i-propyl, and 1'-n-propylindolo(2',3')- derivatives. Slight activity was shown by 6,7-dimethyl and 6,7-diphenyl derivatives(64).

It is of interest that certain antimalarials, such as pyrimethamine, possess antifolic activity. Some antifolics are also potent antimalarials. In the above series of compounds tested by Collier and Phillips there was no correlation between antifolinic activity, as evaluated with Leuconostoc citrovorum, and ability to suppress Plasmodium berghei infections of mice(64).

Greenberg reported that 7-methylpteroylglutamic acid, 9-methylpteroylglutamic acid, 2,4-diaminopteroylaspartic acid, and N¹⁰-methylpteroylglutamic acid completely reversed the antimalarial activity of sulfadiazine against P. gallinaceum in chicks(65).

Many other analogs are capable of growth inhibition in microorganisms which require folic acid in their medium; in general, however, only the 4-amino analogs(amethopterin and aminopterin) have proved to be highly active in mammals, including man(66,67,68).

The lives of mice implanted experimentally with leukemia can be prolonged by the administration of folic acid antagonists, such as amethopterin(69). This provides the experimental basis for the therapeutic use of these drugs in human leukemias, especially the acute leukemias of childhood. Burchenal, as early as 1951, cited many cases of good clinical and hematological remissions, as well as partial remissions, in patients treated with amethopterin(70).

Skipper et al demonstrated in mice that a definite relationship exists between the effectiveness of amethopterin and the size of the neoplastic population at the time of initiation of therapy(71). For L1210 leukemia in mice the effectiveness of amethopterin as a "curative agent" was inversely related to the tumor cell population at the time of initiation of therapy. It is of interest to note that folinic acid, or citrovorum factor, antagonizes this therapeutic effect more potently than does folic acid(72).

If leukemic cells from animals treated with folic acid antagonists are injected into susceptible recipients, increasing tolerance to the action of the antimetabolite develops with successive transfers until the drug eventually exerts no chemotherapeutic action. This same type of refractory state develops in clinical leukemia after repeated courses of amethopterin.

In a ten year old child with lymphosarcoma, studied by Whiteside et al, amethopterin was administered in an oral dose of 2.5 mg daily for seven days, followed by a single dose of 10 mg orally(73). Amethopterin appeared in the cerebrospinal fluid one hour after the last dose, reaching a level of 15 $\mu\text{g}/\text{ml}$ in three hours, and falling to 2 $\mu\text{g}/\text{ml}$ in 18 hours. The serum level of amethopterin before the last "priming" dose was 2 $\mu\text{g}/\text{ml}$; thirty minutes following the last dose it was 900 $\mu\text{g}/\text{ml}$, and by 18 hours it had fallen to 5 $\mu\text{g}/\text{ml}$. It is thus seen that the cerebrospinal fluid drug level declines fairly rapidly after intrathecal administration, so that the concentration present after 24 hours exerts a negligible therapeutic effect. Indeed, Welch has pointed out that the limitation on dosage by the intrathecal route appears to be the rate at which the drug leaves the cerebrospinal fluid to enter the systemic circulation, thereby affecting such sensitive tissues as the bone marrow and the mucosa of the gastrointestinal tract(74).

Five patients with neurological symptoms of leukemia and lymphosarcoma improved markedly on doses of 0.1 to 0.5 mg/kg of intrathecal amethopterin(73). Neurological remissions began one week after the instillation and lasted six weeks. From 14% to 33% of the intrathecal dose was excreted in the urine in a four to six day period.

Chorioncarcinoma, or chorione epithelioma, is an uncommon highly malignant tumor of fetal trophoblastic tissue, occurring in pregnant or postpartum women. The presence of this tumor formerly invoked an almost hopeless outlook for the patient, since metastasis usually has occurred before the primary tumor is discovered. Most women afflicted with this tumor die within one to two years of onset. Chorioncarcinoma also exists as a rare testicular tumor in males and carries a similar prognosis in this instance. The treatment in former years has been wide total hysterectomy and removal of all metastases that can be found and removed surgically. Chorioncarcinoma is not radiosensitive. The elaboration of chorionic gonadotrophin is characteristic of this tumor, and the finding of high titers of this substance in the urine of affected patients is useful in diagnosis(75).

With the introduction of therapy with amethopterin the outlook for patients with this tumor has been somewhat improved(76,77). Hertz et al noted that inhibition of estrogen-induced growth in the chick oviduct and monkey uterus occurred when the animals were maintained on a folic acid deficient diet or treated with antagonists of either folic acid or adenine(78). Li, Hertz, and Spencer studied the effect of amethopterin on urinary excretion of chorionic gonadotrophin and on the clinical course of two patients with chorioncarcinoma and one patient with chorioadenoma destruens, a locally invasive form of chorioncarcinoma(78). The drug was administered in a single initial intravenous dose of 1.0 to 4.5 mg/kg, followed by 2.5 mg/kg at two week intervals, each total biweekly dose being divided into five consecutive daily doses. All three cases showed regression of pulmonary metastases. In one case of chorioncarcinoma each course of drug therapy resulted in a decrease in urinary chorionic gonadotrophin

and a regression of symptoms caused by metastatic involvement. The level of urinary chorionic gonadotrophin dropped from 4 million units/ml before therapy to 500 units/ml at the completion of therapy. No definite evidences of toxicity were found, although the possibility of renal impairment, as measured by frequent determinations of blood urea nitrogen, contraindicates this drug in the presence of renal disease. Although hepatic injury has not been observed, some gastrointestinal toxicity and bone marrow depression has been seen. Decreased levels of chorionic gonadotrophin could not be correlated with the presence of excreted amethopterin.

Li, Spencer, Hertz, and Lubs noted in 1957 that the above three patients showed regression of metastases for 8 to 14 months following amethopterin therapy. Three men with embryonal carcinoma of the testis responded to amethopterin therapy, but the response was less sustained. The marked variability in the spontaneous clinical course of trophoblastic tumors renders therapeutic evaluation difficult. The data presented by these authors constitutes unequivocal quantitative evidence, on the basis of decreased urinary chorionic gonadotrophin titers to values approaching normal, that these tumors are susceptible to amethopterin(79). The dose used by Li et al was from 5 to 10 times the average adult dosage used in acute leukemia; drug toxicity was severe but not life-threatening(80).

Some of the patients reported above are now showing relapses, however, with growth of cancer cells in the lungs. In some cases metastases have been noted in the central nervous system(81). According to Welch, growth of cells in the lungs is attributable to the selection of a drug-resistant strain. The appearance of metastatic growth in the brain has stimulated investigations on intrathecral administration of amethopterin and also on the distribution of this drug throughout the body, in particular its capacity to cross the blood-brain barrier. Relapses in these patients may have been due to failure of the drug to reach the population of tumor cells

in the brain during the early period of growth when the tumor cells were more susceptible to the drug. Intrathecal therapy with amethopterin produces very few toxic effects upon the central nervous system. The limitation of dosage by this route depends upon the rate at which the drug leaves the cerebrospinal fluid to enter the systemic circulation, thereby producing toxic effects on the bone marrow and gastrointestinal tract, tissues previously described as having a special sensitivity to amethopterin.

Li, Nixon, and Freeman, on the basis of work by Nichol and Welch(82), showing that folic acid antagonists inhibit the synthesis of citrovorum factor in liver and also prevent its utilization by tissues in animals, studied the daily excretion of urinary citrovorum factor and correlated this with the tumor response in patients with trophoblastic tumors receiving amethopterin therapy(83). In a 34 year old man with metastatic embryonal carcinoma and chorion-carcinoma to lungs and abdomen, the urinary citrovorum factor activity was at a constant low level before treatment; whereas administration of 25 to 35 mg of amethopterin orally or intramuscularly daily for five days resulted in a fivefold increase in urinary citrovorum factor activity; clinical improvement was not observed, however. A similar effect was observed in a 28 year old female with chorioncarcinoma with pulmonary metastases, although the increase in urinary citrovorum factor activity seemed to diminish toward the end of each course and the rise on subsequent courses was less prominent than the preceding ones; tumor regression was obtained in her case.

It was shown that this increased citrovorum factor activity does not result from tumor breakdown, normal tissue, as the result of drug toxicity, or drug contamination. Nichol and Welch proposed originally that folic acid antagonists have at least two actions: 1)inhibition of conversion of folic acid to folinic acid(citrovorum factor),

and 2) inhibition of utilization of citrovorum factor (if blocked would result in an increase in citrovorum factor in the urine). These authors, when measuring urinary citrovorum factor after amethopterin administration to rats, have observed an increased excretion in some of the rats and have interpreted this as possible displacement of citrovorum factor(82). These cases apparently represent a similar displacement of citrovorum factor in humans by the administered amethopterin.

C. Previous studies on the distribution of administered amethopterin and its permeability through the blood-brain barrier; rationale for synthesis of folic acid analogs.

Fountain studied the distribution of amethopterin in normal mouse tissue following intravenous injection of the drug and found that the serum level was high initially(75 μ g/ml) and fell to zero after four hours. The relative concentrations of amethopterin in various tissues appeared to parallel the folic acid and citrovorum factor content, namely liver, kidney, and spleen in descending order. Amethopterin-like activity was still appreciable in liver and kidney after 24 hours(84).

Whiteside et al studied the distribution of intrathecal amethopterin administered to dogs(73). At a dosage of 1.8 mg/kg it was shown that the initial cerebrospinal fluid drug level of 15 μ g/ml fell to 56 μ g/ml after 43 hours, and that only a trace amount of amethopterin could be detected after 116 hours. Studies in man by Whiteside et al have been described in the previous section.

Studies in man by Freeman indicate that amethopterin is rapidly and totally absorbed after oral administration(85). The peak plasma level, reached after one hour in most cases, was proportional to the dose given. In plasma approximately 50% of the drug was found to be bound to plasma proteins. The distribution was limited to the extracellular space, and no demonstrable storage was observed in the human after repeated small doses. After an intravenous dose of 18 mg

the plasma level quickly rose to 1.5 $\mu\text{g}/\text{ml}$, following which it rapidly fell, so that the concentration was less than 0.5 $\mu\text{g}/\text{ml}$ after three hours. The cerebrospinal fluid drug concentration in the same experiment reached 50 $\mu\text{g}/\text{ml}$ after three hours and did not rise above this value.

After intravenous administration in man the drug was excreted very rapidly, an average of 99% of the administered drug appearing in the urine within 12 hours. In the presence of renal failure, plasma levels were prolonged for a much greater period of time(85).

Wollner, Murphy, and Gordon in 1959 studied the distribution of amethopterin after intravenous injection into dogs, and found that only minute amounts of the drug entered the cerebrospinal fluid. No actual values were given for the cerebrospinal drug levels(86). Cerebrospinal fluid/serum concentration ratios at equilibrium were found to be less than 0.003 in dogs receiving constant intravenous infusions of drug. Intrathecal doses resulted in high concentrations of drug being maintained in the cerebrospinal fluid for longer than 24 hours. The serum concentration of amethopterin fell rapidly after intravenous injection; following intrathecal administration, however, the serum concentration, although lower initially, was maintained for a longer period of time. From 60% to 80% of administered amethopterin was recovered within 48 hours from the urine following intravenous or intrathecal injection. Simultaneous renal clearance studies revealed that amethopterin was excreted at a constant rate approximating that of creatinine.

Freeman has commented on the appearance of a second substance, presumably a metabolite, in the urine of mice, rats, dogs, and all patients examined(except patients with chronic leukemia) following the administration of amethopterin(87). This secondary substance continues to appear in the urine for a period of four to twenty days after cessation of

drug administration.

Freeman has reported that the capacity of amethopterin to cross the blood-brain barrier is extremely low(85). Failure of the drug to attain appreciable levels in the brain is one possible reason for poor results observed in the clinical treatment of cerebral manifestations of leukemia and chorioncarcinoma.

Rationale for synthesis of folic acid analogs.

It was hoped that the introduction of slight modifications in the amethopterin molecule might confer upon it the ability to pass the blood-brain barrier while still retaining chemotherapeutic activity.

It has long been known that, other factors being equal, drugs having a higher lipid solubility penetrate into the brain more rapidly than those with a lower lipoid solubility.

A number of isolated reports hint at the lipoid nature of the blood-brain barrier. Experiments cited by Mayer et al indicate that for foreign compounds the blood-brain barrier behaves as a lipoid membrane, and that the permeability of the blood-cerebrospinal fluid and blood-brain barriers are identical with respect to drugs(12). A widely accepted concept of the cell membrane describes the boundary as a fat-like layer interspersed with small pores. Penetration of such a membrane could occur by several processes:

1)lipid-soluble substances may passively penetrate into the cell by dissolving in the lipid-like phase; 2) non-polar substances, such as water and urea, may pass by free diffusion if the pore size is sufficient to accomodate them; 3)in addition to these physical processes, special transfer mechanisms involving temporary combination with membrane constituents are required to transport inorganic ions, numerous endogenous substrates, and certain foreign compounds, among which antimetabolites are an important example.

Since nerve tissue contains lipids it is often stated that drugs gain access to the central nervous system by virtue of their solubility in brain lipids; this statement is often made in connection with the highly lipid-soluble thio-barbiturates, such as thiopental. There is no indication that lipid-soluble drugs have any special predilection for brain tissue, suggesting that the structural lipids do not have the solubility characteristics of neutral fat. It is presumably the lipoid characteristics of the blood-brain barrier and not of the brain tissue cells which regulate the entrance of drugs into the brain.

Water-soluble organic compounds, such as sucrose, penetrate into the brain very slowly. Highly ionized bases, such as tetraethylammonium and tubocurarine, penetrate with great difficulty. Brodie describes experiments which indicate that the rate of penetration of drugs into the central nervous system is dependent upon the lipid solubility of the uncharged molecule(10).

Preparation of the dimethyl ester of amethopterin was thought to be one method of increasing the lipid solubility without altering appreciably that portion of the molecule which is perhaps more intimately involved in blocking the folic-folinic acid biotransformation(Figure I).

The diethyl ester of amethopterin was also prepared, since this substitution might increase the lipid solubility of the compound over that of the dimethyl ester. Since much more difficulty was encountered in preparing the diethyl ester in a soluble form suitable for injection than was experienced with the dimethyl ester, it was thought that the preparation of higher analogs, such as the dipropyl ester, would engender such difficulties in solubilization in return for a negligible increase in lipid solubility that this line of approach was abandoned.

The introduction of two chlorine atoms into the p-aminobenzoic acid ring of amethopterin is another possible way of increasing lipid solubility. In this case, however, more drastic effects on the "enzymatically active" portion

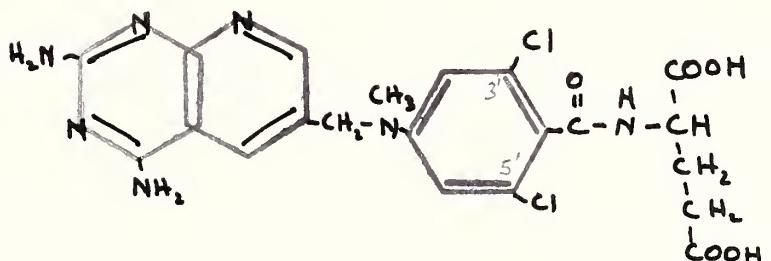
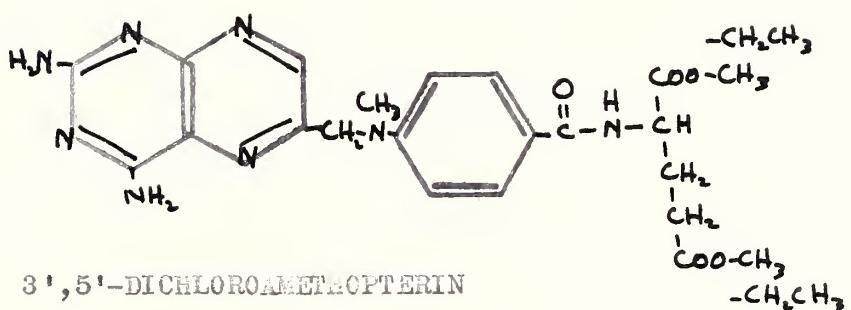
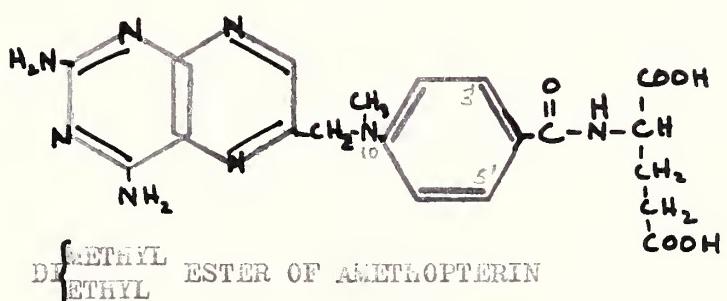
of the molecule might be expected. Furthermore, the addition of a halogen atom to an organic acid can be expected to change the extent of ionization. This is well illustrated by the change conferred upon acetic acid, which is only slightly ionized, by the substitution of three chlorine atoms. Trichloroacetic acid exhibits such a high degree of ionization that it is classified as a strong acid. Rall et al commented on the effect of extent of ionization on degree of penetration of the series of compounds, aniline, barbitone, amidopyrine, and salicylic acid. They noted that the degree of penetration into the brain could be inversely correlated with extent of ionization at pH 7.4. The distribution of certain of these drugs between blood and cerebrospinal fluid could be significantly and predictably altered by induction of alkalosis or acidosis in the experimental animals(1).

If the presence of the chlorine atoms on the p-aminobenzoic acid ring of amethopterin does increase the degree of ionization, the penetration of the compound into the brain might be expected to be hindered. It could be theorized, however, that the bulkiness of the two chlorine atoms present might increase the lipid solubility sufficiently to offset the effect of possibly increased ionization(Figure I).

Goldin et al noted recently that 3',5'-dichloroamethopterin was much more effective than amethopterin in extending the mean survival time of mice implanted with L1210 leukemia(88). Some of the mice tested were presumably cured and did not accept reinoculation with leukemic cells. The antileukemic action of this derivative could be reversed by the concomitant administration of citrovorum factor. Other surviving mice showed partial immunity(89). This drug is being used in clinical trials at the present time(88).

FIGURE I
EXPERIMENTAL DRUGS

AMETHOPTERIN



II. EXPERIMENTAL

A. Materials and methods.

The experimental protocol involved collection of control specimens of plasma and cerebrospinal fluid from dogs, intravenous administration of a known dose of experimental drug, and collection of samples of plasma and cerebrospinal fluid at intervals following the drug injection. The plasma and cerebrospinal fluid samples were then analyzed to determine the drug levels present during each time interval.

Healthy mongrel dogs weighing approximately 10 kg were anesthetized with 30 mg/kg nembutal given intraperitoneally and were maintained in adequate anesthesia during the course of the experiment by the additional administration of 10 mg/kg nembutal as required. Each dog was placed in the right lateral decubitus position with the head and neck well supported and the head moderately flexed. After shaving of the suboccipital area of the neck, cerebrospinal fluid samples were obtained from the cisterna magna by inserting a 50 mm #22 pediatric type lumbar puncture needle posteriorly just below the inferior edge of the occipital bone in the midline, and directing the needle toward the level of the hyoid bone palpated anteriorly(90,91). Forward progress of the needle should be carefully controlled by counter-pressure against the neck; a sensation of decreased resistance, or "give," is experienced when the needle enters the cistern. At this point removal of the stylet usually yields a free flow of cerebrospinal fluid; if this does not occur, the needle should be rotated slightly or inserted 1 mm farther and rechecked. Once a free flow is established the needle is taped in place, remaining in the cistern throughout the duration of the experiment. Serial samples of cerebrospinal fluid are easily collected through this needle; it was not found necessary to use an intraluminal catheter.

About 1.0 ml of cerebrospinal fluid was removed at any one time. All samples containing red blood cells after centrifugation were discarded, since their presence implies a leak in the blood-brain barrier. Samples of blood were taken at intervals throughout the experiment from superficial leg veins. All syringes used for blood collection were first rinsed with saturated sodium oxalate solution; also, 0.1 ml of saturated sodium oxalate solution was added to each 5 ml blood sample collected. All blood samples were centrifuged immediately after collection for 10 minutes at 3000 RPM to remove formed elements, and the clear plasma as well as all cerebrospinal fluid samples were kept in an ice bath until the time of analysis. Since heparin has been demonstrated to quench fluorescence it was not used as an anticoagulant in these experiments. In the event that the initial cisternal tap was grossly or microscopically bloody, the dog was rejected for further use on that day.

After control samples of cerebrospinal fluid and plasma had been obtained and it had been assured that no red blood cells had been found in the cerebrospinal fluid, the required weight of drug was prepared for injection. Folic acid analogs were prepared by dissolving the required weight of recrystallized drug in 6 ml of 0.5N hydrochloric acid, with warming. Water was added to about 25 ml, then 5N sodium hydroxide dropwise to pH 4.0. The pH was never allowed to rise above 7.0 during the procedure; it was found that most folic acid analogs formed a milky suspension unsuitable for injection above pH 4.0. The final volume of solution was about 30 ml.

One gram of sulfadiazine was prepared for administration by dissolving two 0.5 gm tablets of the drug in 10 ml of 3.5% sodium hydroxide, adding water to about 45 ml, then 4N hydrochloric acid dropwise to pH 9.0. The final volume of solution was about 50 ml.

The drug solution to be administered was placed in a suitable syringe and injected over a 60 second period into

the right anterior foreleg vein. This vein was avoided during subsequent blood sampling. Samples of blood and cerebrospinal fluid were obtained at 15 minutes, 60 minutes, and 180 minutes after the injection of drug, and were treated as previously described for the control samples. In a few experiments, samples were obtained at 15 minutes, 30 minutes, and 60 minutes after drug administration. When all four samples of plasma and cerebrospinal fluid had been accumulated, they were analyzed at the same time for the experimental drug. There was no difficulty in maintaining the position of the needle in the cisterna magna during the three-hour course of each experiment. All dogs were given 300,000 units of benzathine penicillin prophylactically; they recovered quickly from the anesthesia and remained in good condition following each experiment.

Sulfadiazine was determined by the method of Bratton and Marshall, which involves diazotization of a deproteinized solution of the drug and coupling with N-naphthylethylenediamine to yield a colored product(92). The procedure used was as follows:

Procedure for Sulfadiazine: To 1 ml of plasma or cerebrospinal fluid, add 1 ml of 15% trichloroacetic acid and centrifuge to remove protein. To 0.5 ml of the supernatant is added 1.0 ml of 0.1% sodium nitrite; after 3 minutes, 1.0 ml of ammonium sulfamate is added. After 3 minutes 1.0 ml of the dye, N-naphthylethylenediamine, is added. After 20 minutes the optical density of this product is determined at 540 μm in a Beckman Spectrophotometer.

A standard curve for sulfadiazine was exactly linear at concentrations of 1, 5, and 10 $\mu\text{g}/\text{ml}$.

Amethopterin and its analogs were determined by a photofluorometric method modified after that of Freeman(93,94). The original method as outlined by Freeman is as follows:

Procedure for Amethopterin: Collect whole blood in oxalated tubes and separate plasma by centrifugation. Siphon plasma into a clean test tube. To 1 ml of plasma add 7 ml of distilled water and mix. Add 2 ml of 15% trichloroacetic acid dropwise, with continuous stirring. Centrifuge. To 5 ml of the supernatant add 1.0 ml of 3.24% sodium hydroxide. Add 0.1 ml of 5M acetate buffer pH 5 and mix.

Measure fluorescence(A). Add 0.05 ml of 4% potassium permanganate. Wait 5 minutes. Add 0.1 ml 3% hydrogen peroxide and mix; wait 3 minutes and measure fluorescence(B).

Reading (B) minus reading (A) yields the fluorescence increment; this is corrected by means of a blank determination. The corrected increment is converted to concentration of amethopterin by means of a standard curve.

Since the relationship between fluorescence increment and concentration of drug was not found to be linear using Freeman's original method (Table I), modification of the method was necessary.

Using sodium hydroxide in a concentration of 3.24% as suggested by Freeman, it was noted that the final pH of the solution was 8.4 rather than 5.0, as it should be when reading (B) is done. In addition, quenching of fluorescence was observed at pH 8.4 (Table II). Amethopterin standards run without the addition of trichloroacetic acid or sodium hydroxide had a final pH of 4.8 and a significant fluorescence increment, whereas the addition of trichloroacetic acid and sodium hydroxide depressed the final pH to 3.4 and resulted in a negligible fluorescence increment.

An optimum pH for the procedure was determined by titrating 1 microgram samples of amethopterin with sodium hydroxide, and determining the fluorescence readings at increments of 0.1 pH unit (Table III). It was apparent that a pH of 5.0 represented an optimum value for the analysis, since the fluorescence is high at this point and the pH still does not approach neutrality, with the possible precipitation of manganese dioxide at alkaline pH values.

Accordingly, an electrometric titration was carried out to determine the concentration of sodium hydroxide required in the analysis to achieve a final pH of 5.0. The titration required 8.4 ml of 0.1064N sodium hydroxide, or 0.89376 milliequivalent of base, to bring the pH to 5.0. This represents 1.0 ml of 3.6% sodium hydroxide, rather than

TABLE I
STANDARD CURVE USING FREEMAN'S ORIGINAL METHOD

<u>Concentration of Amethopterin</u>	<u>Fluorescence Increment</u>
100 μ g/ml	0.05
1000 μ g/ml	0.46
10,000 μ g/ml	3.96

Deproteinized amethopterin standards were treated with 1 ml of 3.24% sodium hydroxide and 0.1 ml of 5M acetate buffer pH 5.0. After reading (A) was taken, the solutions were treated with 0.05 ml of 4% potassium permanganate and 0.1 ml of 3% hydrogen peroxide, and reading (B) was taken.

TABLE II

EFFECT OF ADDITION OF 3.24% SODIUM HYDROXIDE AND TRICHLOROACETIC ACID ON FINAL pH AND FLUORESCENCE INCREMENT (EACH VALUE REPRESENTS TWO EXPERIMENTS)

<u>Amethopterin Concentration</u>	<u>Buffer</u>	<u>TCA+NaOH added</u>	<u>Increment</u>	<u>Final pH</u>
0	0.1 ml	-	-0.01	4.3
1 μ g/ml	0.1 ml	-	+0.41	4.3
0	0.1 ml	+	-0.02	3.4
1 μ g/ml	0.1 ml	+	+0.15	3.3

Amethopterin standards in water solution were treated with 0.1 ml of buffer (controls), or 0.1 ml of buffer + 1 ml 15% trichloroacetic acid (TCA) + 1 ml 3.24% sodium hydroxide (experimental). After reading (A), 0.05 ml of 4% potassium permanganate and 0.02 ml of 3% hydrogen peroxide were added, and reading (B) was taken.

TABLE III

DETERMINATION OF OPTIMUM pH FOR ANALYSIS

<u>pH</u>	<u>Fluorescence Increment</u>
4.5	0.28
4.6	0.47
4.7	0.62
4.8	0.64
4.9	0.81
5.0	1.01
5.1	1.26

To a 1 μ g sample of amethopterin in water solution were added 1 ml of 15% trichloroacetic acid and 1 ml of 3.5% sodium hydroxide; 0.1 ml of 5M acetate buffer pH 5.0 was then added. After reading (A) was taken 0.05 ml of 4% potassium permanganate and 0.02 ml of 3% hydrogen peroxide were added. Reading (B) was taken. Reading (B) minus reading (A) yields the fluorescence increment shown above. The solution was then titrated electrometrically with 0.1 N sodium hydroxide in increments of 0.1 pH unit. A fluorescence reading was taken at each pH increment.

3.24% as suggested by Freeman. The use of this concentration of sodium hydroxide resulted in a significant fluorescence increment and the desired end pH of 5.0 to 5.1 (Table IV).

After the step in the analysis involving the addition of 0.1 ml of 3% hydrogen peroxide, a brown precipitate of manganese dioxide was occasionally observed in some of the plasma samples; values obtained with such samples were inaccurate. The precipitate could be dissolved by the addition of another 0.1 ml of 3% hydrogen peroxide. The use of 0.2 ml of 3% hydrogen peroxide, as opposed to 0.1 ml, has no effect on the fluorescence increment (Table V). The routine use of 0.2 ml of 3% hydrogen peroxide gave stable reproducible values, and no difficulties were encountered with manganese dioxide precipitates.

In addition, fluorescence increment values obtained with water solutions of amethopterin were not comparable with those obtained with plasma solutions of the same drug. It was discovered that dilution of plasma samples 1:10 before analysis resulted in experimental values closely approximating those obtained in standard solutions of amethopterin in water. Fluorescence increment values with undiluted plasma averaged 1.6 times those obtained with plasma diluted 1:10 or with water, using identical concentrations of amethopterin (Table VI).

Amethopterin could be estimated to \pm 16% in water solution and plasma diluted 1:10, when known quantities were added to each. It could be detected accurately in concentrations as low as 40 $\mu\text{g}/\text{ml}$ (Table VII). Two cerebrospinal fluid standards at 80 $\mu\text{g}/\text{ml}$ were accurate within 0% and 25% respectively. The method of determination finally adopted was as follows:

ANALYTICAL METHOD FOR THE DETERMINATION OF FOLIC ACID ANALOGS IN PLASMA AND CEREBROSPINAL FLUID

Procedure: To 0.1 ml of plasma or 1.0 ml of cerebrospinal fluid, add 7.9 ml or 7.0 ml respectively of distilled water and mix thoroughly. Add 2.0 ml of 15% trichloroacetic acid dropwise, with continuous stirring. The mixture is then centrifuged at 10,000 RPM for 15 minutes to remove the precipitated protein. To 5 ml of

TABLE IV

RESULTS OF ANALYSIS USING 3.6% SODIUM HYDROXIDE AND pH 5.0 BUFFER

<u>Ametopterin Concentration</u>	<u>Fluorescence increment</u>	<u>pH</u>
0	0	5.0
0	-0.02	5.1
1 μ g/ml	+1.45	5.0
1 μ g/ml	+1.61	5.0

To deproteinized solutions of ametopterin were added 1 ml of 3.6% sodium hydroxide and 0.1 ml of 5M acetate buffer pH 5.0. After reading (A) was taken, 0.05 ml of 4% potassium permanganate and 0.02 ml of 3% hydrogen peroxide were added. Reading (B) and the final pH were then determined.

The difference in fluorescence increment values in tables II and IV is due to the different concentrations of sodium hydroxide used in the two instances. In addition daily fluctuations in the sensitivity of the spectrofluorophotometer were observed; daily standards were thus necessary.

TABLE V

EFFECT OF DIFFERENT VOLUMES OF 3% HYDROGEN PEROXIDE ON FLUORESCENCE INCREMENT

<u>Ametopterin Concentration</u>	<u>Volume of Peroxide</u>	<u>Fluorescence increment</u>
1 μ g/ml	0.1 ml	+0.44
1 μ g/ml	0.1 ml	+0.42
1 μ g/ml	0.2 ml	+0.45
1 μ g/ml	0.2 ml	+0.42

To standard solutions of ametopterin in water was added 0.1 ml of 5M acetate buffer pH 5.0. After reading (A), 0.05 ml of 4% potassium permanganate and either 0.1 or 0.2 ml of 3% hydrogen peroxide were added. Reading (B) was then determined.

TABLE VI

COMPARISON OF FLUORESCENCE INCREMENT VALUES OBTAINED
WITH UNDILUTED PLASMA, PLASMA DILUTED 1:10, AND WATER

<u>Amethopterin Concentration(mg/ml)</u>	<u>Medium</u>	<u>Fluorescence increment</u>
80	water	0.08
80	undiluted plasma	0.14
80	undiluted plasma	0.14
80	plasma 1:10	0.08
80	plasma 1:10	0.08
160	water	0.17
160	undiluted plasma	0.21
160	undiluted plasma	0.25
160	plasma 1:10	0.14
160	plasma 1:10	0.15
320	water	0.35
320	undiluted plasma	0.54
320	undiluted plasma	0.50
320	plasma 1:10	0.33
320	plasma 1:10	0.32

Deproteinized standards were treated with 1.0 ml of 3.6% sodium hydroxide and 0.1 ml of 5M acetate buffer pH 5.0. After reading (A) was taken, 0.05 ml of 4% potassium permanganate and 0.02 ml of 3% hydrogen peroxide were added, and reading (B) was taken(modified method of analysis).

TABLE VII

RECOVERY OF AMETHOPTERIN FROM WATER,
CEREBROSPINAL FLUID, AND 1:10 PLASMA

<u>Medium</u>	<u>Quantity present(mug/ml)</u>	<u>Quantity recovered(mug/ml)</u>	<u>% Difference</u>
water	40	40	0 %
water	40	40	0 %
water	80	80	0 %
water	80	70	-13 %
water	80	80	0 %
water	80	67	-16 %
water	80	80	0 %
water	80	93	+16 %
water	80	70	-13 %
water	80	90	+13 %
water	160	170	+ 7 %
water	320	320	0 %
water	320	258	-18 %
water	320	305	- 5 %
water	320	294	- 8 %
water	320	346	+ 8 %
water	1000	990	- 1 %
plasma	80	88	+10 %
plasma	80	70	-13 %
plasma	80	80	0 %
plasma	160	150	- 7 %
plasma	160	160	0 %
plasma	320	330	+ 3 %
plasma	320	320	0 %
plasma	320	312	- 3 %
plasma	1000	1000	0 %
plasma	1000	1160	+16 %
plasma	1000	1120	+12 %
cerebrospinal fluid	80	80	0 %
cerebrospinal fluid	80	100	+25 %

the supernatant in a separate tube add 1.0 ml of 3.6% sodium hydroxide and 0.1 ml of 5M acetate buffer pH 5.0. After thorough mixing the fluorescence(reading A) is determined in a spectrofluorophotometer with activation wavelength 370 m μ and fluorescence wavelength 450 m μ . To the mixture is then added 0.05 ml of 4% potassium permanganate, and the solution is mixed thoroughly and allowed to stand for five minutes. After this period of time has elapsed, 0.2 ml of freshly prepared 3% hydrogen peroxide is added to remove the excess permanganate. The solution is mixed well and allowed to stand for three minutes. The fluorescence is again determined as before(reading B).

Interpretation: Same as Freeman's method(page 24).

Comments on the experimental procedure:

1. The activating wavelength is variously given in the literature as between 280 m μ and 370 m μ , and the fluorescent wavelength between 450 m μ and 470 m μ (85,94). Preliminary experiments indicated that the optimum wavelengths were 370 m μ activating and 450 m μ fluorescent.
2. All solutions should be freshly made and carefully protected from air, since the increment is constant only if the pH is held within rather close limits. The 3% hydrogen peroxide is made just before use by 1:10 dilution of 30% hydrogen peroxide. The buffer is prepared by adding 70 ml of 5M sodium acetate solution to 30 ml of 5M acetic acid, then adding glacial acetic acid dropwise until a 1:10 dilution of the buffer has a pH of 5.0.
3. The second fluorescence reading(B) should be taken within 10 seconds after filling the cuvette with the solution, since oxygen bubbles are released at this point, causing a marked increase in the reading. If more time is needed, the cuvette should be gently tapped to eliminate bubbles.
4. Standard solutions of amethopterin at various concentrations were run with each experimental analysis, since there were slight day to day fluctuations in the experimental readings obtained for given concentrations of drug.
5. If any solutions appear cloudy after centrifugation, they should be re-centrifuged, since even the slightest turbulence causes a marked increase in the fluorescence reading.

6. Not more than 12 to 15 samples should be run at one time, since the fluorescence may decrease if the solutions are allowed to stand for too long a period of time before reading.
7. Toward the latter part of the work a new Xenon light source was installed in the spectrofluorophotometer, resulting in much higher sensitivity readings for given concentrations of amethopterin. At this time, concentrations as low as 10 μg could be detected.

The standard curve for conversion of fluorescence increment to concentration of amethopterin was prepared by dissolving 10.00 \pm 0.001 mg of drug (weighed on a microbalance) in 100 ml of distilled water; this solution contained 100 $\mu\text{g}/\text{ml}$. Reference standards were prepared by diluting this stock solution 1:10, 1:100, 1:1,000, and 1:10,000. The standard curve obtained by analysis of these solutions according to the method described above yields an exactly linear plot of fluorescence increment vs concentration (Table VIII; Figure II).

Standards run with each daily experiment were checked against the standard curve for verification of the correctness of the daily experimental procedure before being used to convert increment readings to concentrations of drug.

The results of analysis of the various analogs are reported in terms of amethopterin concentration. Drugs used experimentally were amethopterin (4-amino-N¹⁰-methylpteroyl-glutamic acid)*, and dimethyl and diethyl esters of amethopterin, ** and 5',5'-dichloroamethopterin* (Figure I).

* commercial samples

** synthesized by A. Eisenfeld

TABLE VIII
DATA FOR STANDARD CURVE

<u>Ametopterin Concentration(mg/ml)</u>	<u>Increment</u>	<u>K(Increment/Concentration)</u>
0	0	
10	0.02	0.002
100	0.19	0.0019
1000	1.94	0.0019
10000	18.94	0.0019

FIGURE II

STANDARD CURVE FOR AMETHOPTERIN

Log amethopterin concentration in
μg/ml vs log fluorescence increment

LOG FLUORESCENCE INCREMENT

+2

+1

0

-1

-2

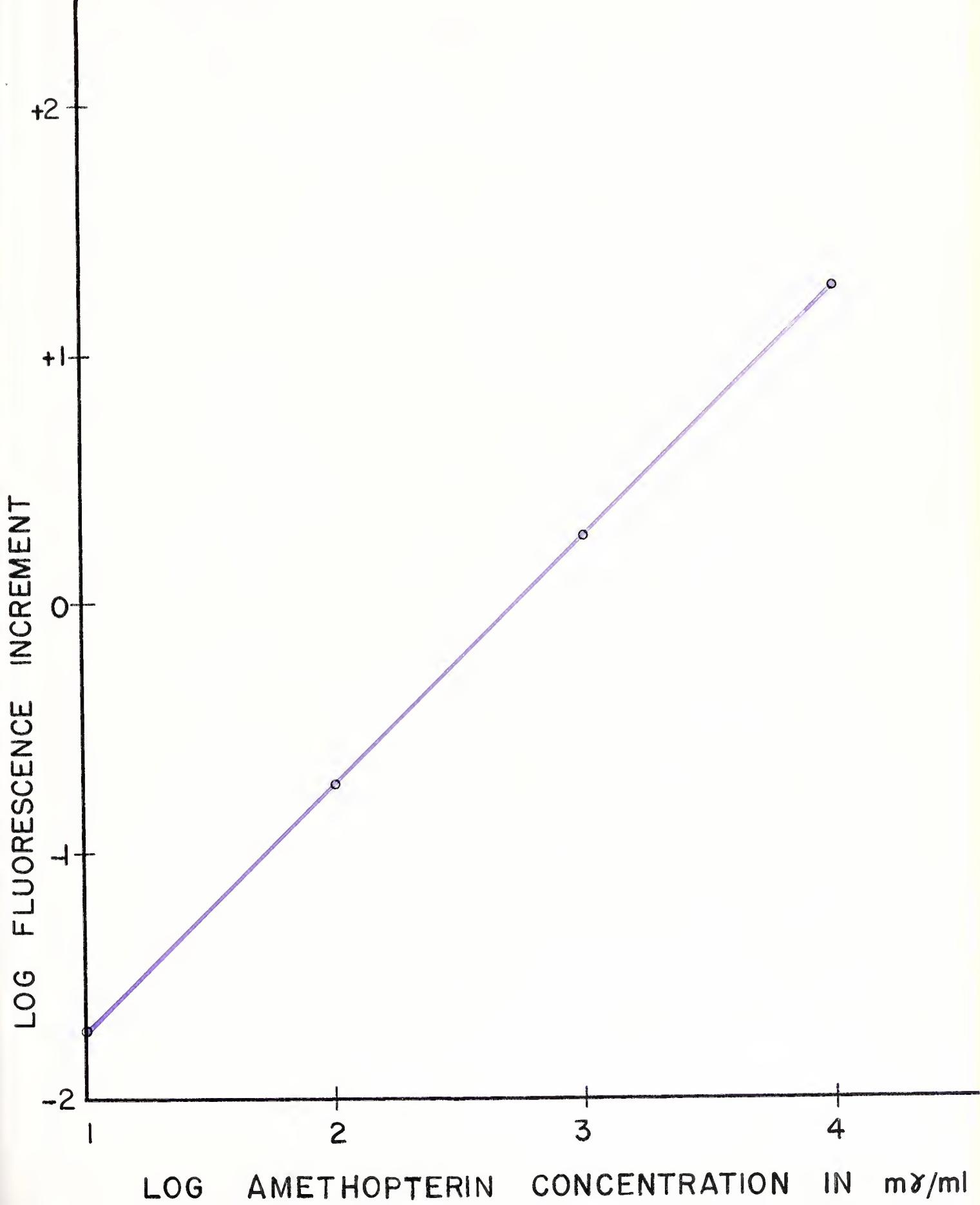
1

2

3

4

LOG AMETHOPTERIN CONCENTRATION IN m γ /ml



B. Results and discussion.

No untoward reactions were observed to any of the drugs used, except that for about ten minutes following injection of amethopterin or its analogs the degree of anesthesia lightened, occasionally requiring the administration of additional nembutal to maintain adequate anesthesia.

In order to test the validity of the experimental method and the method of sampling, sulfadiazine, a drug known to penetrate the blood-brain barrier, was administered in high dosage(100 mg/kg) intravenously(1,95). This drug was found to penetrate quickly into the cerebrospinal fluid, being detectable at levels of 1.2 $\mu\text{g}/\text{ml}$ after 15 minutes, 7.7 $\mu\text{g}/\text{ml}$ after 60 minutes, and 15.6 $\mu\text{g}/\text{ml}$ after 180 minutes(Table IX). The plasma concentration of this drug over the same time interval was decreasing from 48 $\mu\text{g}/\text{ml}$ to 31 $\mu\text{g}/\text{ml}$. It is thus apparent that the experimental procedure described previously provides a valid screening method in testing experimental drugs for penetration into the cerebrospinal fluid.

Amethopterin was seen to penetrate very slightly into the cerebrospinal fluid when injected intravenously in a dosage of 5 mg/kg(Table IX). This confirms the work of Wollner et al, although they did not state the exact level of drug found in the cerebrospinal fluid in their experiments. When amethopterin was administered in a dosage of 10 mg/kg, the drug was undetectable in the cerebrospinal fluid after 15 minutes. The trace level seen with the lower dosage is at the lower limit of detectability of the analytical method, and it may well be that slight variations in the procedure of administration of drug and/or analysis may account for the failure to find the drug in the cerebrospinal fluid at the higher dosage level.

The rationale for the preparation of the dimethyl ester of amethopterin has already been described. In a preliminary experiment, the dimethyl ester of amethopterin was found to penetrate into the cerebrospinal fluid to the

extent of 32 $\mu\text{g}/\text{ml}$ 30 minutes after administration of 5 mg/kg . When the experiment was repeated at a dosage of 10 mg/kg , however, the compound was not detectable in the cerebrospinal fluid (Table IX).

The diethyl ester was also administered to dogs in a similar dosage, and failed to penetrate into the cerebrospinal fluid at the same that appreciable plasma concentrations were present.

When 3',5'-dichloroamethopterin was injected intravenously into dogs in a dosage of 10 mg/kg , slight but measurable amounts were detectable in the cerebrospinal fluid after 60 minutes, in repeated experiments. This finding supports the clinical trials presently being conducted with this drug.

It was initially planned to test other analogs of folic acid for penetration through the blood-brain barrier, but these proposed experiments had to be abandoned, since other compounds were unavailable.

The rapid decline in plasma levels seen during the first three hours after administration of these drugs is in agreement with the work previously published by Fountain, in which a high initial plasma level of amethopterin fell to almost zero after four hours.

Failure of the dimethyl and diethyl esters to penetrate into the cerebrospinal fluid in some of these experiments may have been due to rapid hydrolysis of the compounds, particularly by the liver. Preliminary observations indicated that rapid hydrolysis of dimethylamethopterin was observed in blood, brain, and especially the liver.

These experiments do not represent an attempt to measure the rates of penetration of these drugs into the cerebrospinal fluid, since a constant plasma level was not maintained during the course of each experiment. These experiments were designed as a screening method useful in finding a compound which would penetrate the cerebrospinal fluid readily and in high concentration. Had such a compound been discovered, rate studies would have been carried out.

TABLE IX

PLASMA AND CEREBROSPINAL FLUID DRUG LEVELS AFTER
INTRAVENOUS ADMINISTRATION INTO DOGS

Drug	Dose (mg/kg)	Plasma Levels (μg/ml)				CSF Levels (μg/ml)			
		0	15	60	180 (min)	0	15	30	60
Amethopterin	5					0	trace	0	0
Amethopterin	10	0	16.2	9.5	3.6	0	0	*	*
Dimethylamethopterin	5					0	trace	32	trace
Dimethylamethopterin	10	0	5.5	1.8	1.2	0	0	0	0
Dietnylethopterin	10	0	5.0	1.6	*	0	0	0	0
Dichloroamethopterin	10	0	5.2	1.4	0	0	16	32	16
Dichloroamethopterin	10	0	11.0	3.6	0.8	0	0	30	10
Sulfadiazine	100	0	48	41	31	0	1200	7700	15,600

*Not determined; technical error

The study of penetration of drugs into the brain is presently a fertile field for experimental investigation. It is hoped that the analytical methods and screening procedures presented here will enable future investigators to test experimental analogs of folic acid, as well as other newly developed chemotherapeutic agents, for penetration through the blood-brain barrier.

III. SUMMARY AND CONCLUSIONS

1. Folic acid analogs can be determined with quantitative accuracy in blood and cerebrospinal fluid, in concentrations as low as 40 $\mu\text{g}/\text{ml}$. The method of analysis presented yields a constant linear relationship between fluorescence increment and concentration of compound between the range 40 $\mu\text{g}/\text{ml}$ to 10 $\mu\text{g}/\text{ml}$. The experimental error of the analytical method was found to be $\pm 16\%$ (Table VII). Concentrations in the range of 5 $\mu\text{g}/\text{ml}$ to 40 $\mu\text{g}/\text{ml}$ are detectable but the values obtained are subject to a higher degree of error.
2. A method is presented for the easy removal of samples of cerebrospinal fluid from dogs without trauma or contamination of the samples by blood leakage.
3. An experimental protocol is described for screening proposed chemotherapeutic agents, in order to determine penetration of the agents from the blood into the cerebrospinal fluid. The validity of this procedure is verified by demonstration of the rapid penetration of sulfadiazine, a compound known to penetrate the blood-brain barrier easily.
4. Amethopterin, dimethylamethopterin, diethylamethopterin, and 3',5'-dichloroamethopterin were infused intravenously over a period of one minute in a dosage of 10 mg/kg into healthy dogs. Plasma levels were determined at 15 minutes, 60 minutes, and 180 minutes after infusion of drug. Highest plasma levels measured for all four compounds were attained at the 15 minute time interval, with a gradually decreasing plasma concentration over the next 165 minutes.
5. Amethopterin penetrated into the cerebrospinal fluid in trace levels (about 7 $\mu\text{g}/\text{ml}$) 15 minutes after intravenous infusion of 5 mg/kg of drug.

6. Esterification of the glutamic acid moiety of amethopterin improves the penetration over that of the parent compound; 30 minutes after intravenous administration of 5 mg/kg of dimethylamethopterin, 32 μ g/ml were found in the cerebrospinal fluid. Neither dimethylamethopterin nor diethylamethopterin penetrated into the cerebrospinal fluid at a dosage of 10 mg/kg. Possible reasons for these findings are discussed.
7. The compound 3',5'-dichloroamethopterin was found to penetrate into the cerebrospinal fluid, being detectable there in low concentrations. At a dose of 10 mg/kg of drug, the maximum concentration of drug reached in the cerebrospinal fluid was 30 μ g/ml 60 minutes after infusion of drug. The plasma level of drug at this time interval was 3.6 μ g/ml.
8. Various factors which influence the penetration of drugs into the brain are reviewed. It appears essential that the medicinal chemist synthesize drugs with a high organic solvent/water distribution ratio at pH 7.4, in order to insure ready penetration into the central nervous system.

IV. BIBLIOGRAPHY

1. Rall, D.P., Stabenan, J.R., and Zubrod, C.G. Distribution of drugs between blood and cerebrospinal fluid: general methodology and effect of pH gradients. *Jour. Pharm. Exp. Therap.* 125, 185(1959).
2. Fulton, John F. "A Textbook of Physiology." Saunders, Philadelphia, 1955.
3. Day, R.L. Inhibition of brain respiration in vitro by bilirubin. Reversal of inhibition by various means. *Proc. Soc. Expt. Biol. Med.* 85, 261(1954).
4. Rodriguez. *Jour. Comp. Neurol.* 102, 27(1955).
5. McIlwain, Henry. "Biochemistry and the Central Nervous System." Little, Brown and Co., Boston, 1955.
6. Waelsch. *Proc. Int. Neurochem. Symposium.* 1, 187(1955).
7. Richter, Derek. "Metabolism of the Nervous System." Pergamon Press, New York, 1957.
8. Bakay, Louis. "The Blood-brain Barrier." Charles Thomas, Springfield, Illinois, 1956.
9. "CIBA Foundation Symposium on Cerebrospinal Fluid; Production, circulation, and absorption." Little, Brown and Co., Boston, 1958.
10. Brodie, Bernard B., and Hogben, C.A.M. Some physico-chemical factors in drug action. *Jour. Pharmacy and Pharmacology.* 9, 345(1957).
11. Grinker, Roy R., Bucy, Paul C., and Sahs, Adolph L. "Neurology." Charles Thomas, Springfield, Illinois, 1960.
12. Mayer, S., et al. Kinetics of penetration of drugs and other foreign compounds into cerebrospinal fluid and brain. *Jour. Pharm. Exp. Therap.* 127, 205(1959).
13. Netter, Frank H. "The Ciba Collection of Medical Illustrations. Vol. I: The Nervous System." Ciba, New Jersey, 1953.
14. Wallace, G.B., and Brodie, B.B. On the source of the cerebrospinal fluid: the distribution of bromide and iodide throughout the central nervous system. *Jour. Pharm. Exp. Therap.* 70, 418(1940).
15. McIlwain, Henry. "Chemotherapy and the Central Nervous System." Little, Brown and Co., Boston, 1957.
16. Lang, Edward. Some Observations of the Ultrastructure of Adult Mammalian Brain Cells in Tissue Culture. M.D. Dissertation, Yale University, 1960.

17. Clemente, C.D., and Holst, E.A. Pathological changes in neurons, neuroglia, and blood-brain barrier induced by X-irradiation of heads of monkeys. *Arch. Neurol. and Psychiatry.* 71, 66(1954).
18. Cushing, Harvey. "Studies in Intracranial Physiology and Surgery." Oxford Univ. Press, London, 1926.
19. Wislocki, G.B., and Leduc, E.H. Vital staining of the hematoencephalic barrier by silver nitrate and trypan blue, and cytologic comparisons. *Jour. Comp. Neurol.* 96, 371(1952).
20. White, A., et al. "Principles of Biochemistry." McGraw-Hill, New York, 1959.
21. Himwich, H.E. "Brain Metabolism and Cerebral Disorders." Williams and Wilkins Co., Baltimore, 1951.
22. Geiger, A., et al. Effect of blood constituents on uptake of glucose and on metabolic rate of the brain in perfusion experiments. *Am. Jour. Physiol.* 177, 138(1954).
23. Tschirgi, R.D., et al, in "The Biology of Mental Health and Disease." Millbank Memorial Fund, Hoeber, New York, 1952.
24. Abood, L.G., et al in McElroy and Glass(Eds). "Phosphorous Metabolism." Vol. 2. Johns Hopkins Press, Baltimore, 1952.
25. Wang, J.C. Penetration of radioactive sodium and chloride into cerebrospinal fluid and aqueous humor. *Jour. Gen. Physiol.* 31, 259(1948).
26. Manery, J.F., in "The Biology of Mental Health and Disease." Millbank Memorial Fund, Hoeber, New York, 1952.
27. Thomas and McIlwain. *Jour. Neurochem.* 1, 1(1956).
28. Friedemann, Ulrich. Blood-brain barrier. *Physiol. Rev.* 22, 125(1942).
29. Key, A., and Retzius, G. Studier i nerusystemetz anatomi med ire taflor. Norstedt and Söner, Stockholm, 1872.
31. Wills, L., and Bilimoria, H.S. Studies in pernicious anaemia of pregnancy: Part V. Production of macrocytic anaemia in monkeys by deficient feeding. *Indian Jour. Med. Research.* 20, 391(1932).
30. Wills, L. Treatment of "pernicious anaemia of pregnancy" and "tropical anaemia" with special reference to yeast extract as a curative agent. *Brit. Med. Jour.* 1, 1059(1931).

32. Wills, L.; Clutterbuck, P.W.; and Evans, B.D.F. A new factor in the production and cure of macrocytic anaemias and its relation to other haematopoietic principles curative in pernicious anaemia. *Biochem. Jour.* 31, 2136(1937).
33. Day, P.L.; Langston, W.C.; and Shukers, C.F. Leukopenia and anemia in the monkey resulting from vitamin deficiency. *Jour. Nutrition.* 9, 637(1935).
34. Stokstad, E.L.R., and Manning, P.D.V. Evidence of a new growth factor required by chicks. *Jour. Biol. Chem.* 125, 687(1938).
35. Hogan, A.G., and Parrott, E.M. Anemia in chicks caused by a vitamin deficiency. *Jour. Biol. Chem.* 132, 507(1940).
36. Snell, E.E., and Peterson, W.H. Growth factors for bacteria. X. Additional factors required by certain lactic acid bacteria. *Jour. Bact.* 39, 273(1940).
37. Stokstad, E.L.R. Some properties of a growth factor for Lactobacillus casei. *Jour. Biol. Chem.* 149, 573(1943).
38. Mitchell, H.K.; Snell, E.E.; and Williams, R.J. The concentration of "Folic Acid." *Jour. Am. Chem. Soc.* 63, 2284(1941).
39. Pfiffner, J.J., et al. Isolation of the antianemia factor (Vitamin B_c) in crystalline form from liver. *Science.* 97, 404(1943).
40. Hutchings, B.L., et al. Relation of a growth factor required by Lactobacillus casei to the nutrition of the chick. *Jour. Biol. Chem.* 140, 681(1941).
41. Day, P.L., et al. The successful treatment of Vitamin M deficiency in the monkey with highly purified Lactobacillus casei factor. *Jour. Biol. Chem.* 157, 423(1944).
42. Hutchings, B.L., et al. Isolation of a new Lactobacillus casei factor. *Science.* 99, 371(1944).
43. Angier, R.B., et al. The structure and synthesis of the liver L. casei factor. *Science.* 103, 667(1946).
44. Goodman, Louis S., and Gilman, Alfred. "The Pharmacological Basis of Therapeutics." Macmillan, New York, 1956.
45. Nichol, C.A. On the metabolic alteration of ppteroylglutamic acid. *Proc. Soc. Exp. Biol. Med.* 83, 167(1953).
46. Nichol, C.A., and Welch, A.B. Synthesis of citrovorum factor from folic acid by liver slices; augmentation by ascorbic acid. *Proc. Soc. Exp. Biol. Med.* 74, 52(1950).

47. Welch, A.D., and Nichol, C.A. Water-soluble vitamins concerned with one and two carbon intermediates. *Ann. Rev. Biochem.* 21, 683(1952).
48. Welch, A.D., and Heinle, R.W. Hematopoietic agents in macrocytic anemias. *Pharmacol. Rev.* 3, 345(1951).
49. Franklin, A.L., et al. Acceleration of pteroylglutamic acid in mice and chicks by a chemical antagonist. *Proc. Soc. Exp. Biol. Med.* 65, 368(1947).
50. Heinle, R.W., and Welch, A.D. Experiments with pteroylglutamic acid and pteroylglutamic acid deficiency in human leukemia. *Jour. Clin. Invest.* 27, 539(1948).
51. Cosulich, D.B., and ¹⁸Smith, J.A., Jr. Analogs of pteroylglutamic acid. I. N¹⁰-Alkylpteroic acid and derivatives. *Jour. Am. Chem. Soc.* 70, 1922(1948).
52. Hultquist, M.E., et al. Analogs of pteroylglutamic acid. II. 9-methylpteroylglutamic acid and derivatives. *Jour. Am. Chem. Soc.* 71, 619(1948).
53. Seeger, D.R., et al. Analogs of pteroylglutamic acid. III. 4-amino derivatives. *Jour. Am. Chem. Soc.* 71, 1753(1949).
54. Wright, D.B., et al. Analogs of pteroylglutamic acid. IV. Replacement of glutamic acid by other amino acids. *Jour. Am. Chem. Soc.* 71, 3014(1949).
55. Roth, B., et al. Analogs of pteroylglutamic acid. V. 4-alkylamino derivatives. *Jour. Am. Chem. Soc.* 72, 1914(1950).
56. Cosulich, D.B., et al. Analogs of pteroylglutamic acid. VI. 3',5'-dihalopteroyl derivatives. *Jour. Am. Chem. Soc.* 73, 2554(1951).
57. Roth, B., et al. Analogs of pteroylglutamic acid. VII. 2-alkylamino derivatives. *Jour. Am. Chem. Soc.* 73, 2864(1951).
58. Roth, B., et al. Analogs of pteroylglutamic acid. VIII. 4-alkoxy derivatives. *Jour. Am. Chem. Soc.* 73, 2869(1951).
59. Cosulich, D.B., et al. Analogs of pteroylglutamic acid. IX. Derivatives with substituents on the benzene ring. *Jour. Am. Chem. Soc.* 75, 4675(1953).
60. Cosulich, D.B., et al. Nitrosoformyltetrahydropteroic amino acids. U.S. Pat. 2,631,150. *Chem. Abs.* 48, 4598g(1954).
61. Erickman, J.A., Jr., and Roth, B. Tetrahydro compounds with N¹⁰-formylpteroylamino acid nucleus. *Brit. Pat.* 708,304. *Chem. Abs.* 49, 4733b(1955).

62. Brockman, J.A., Jr., and Roth, B. Di- and tetrahydro-formylpteroic acids. U.S. Pat. 2,739,966. Chem. Abs. 50, 10802c(1958).
63. Goldin, A., et al. Comparison of the relative effectiveness of folic acid congeners against advanced leukemias in mice. Jour. Nat. Cancer Inst. 19, 1183(1957).
64. Collier, H.O.J., and Phillips, M. Some 2,4-diaminopteridines as folinic acid antagonists. Nature. 174, 180(1954).
65. Greenberg, J. Effects of analogues of folic acid on the activity of sulfadiazine against Plasmodium gallinaceum. Exper. Parasitology. 3, 351(1954).
66. Petering, H.G. Folic acid antagonists. Physiol. Rev. 32, 197(1952).
67. Burchenal, J.H. Folic acid antagonists, in "Symposium on antimetabolites, their modes of action and therapeutic implication." National Vitamin Foundation, New York, 1955.
68. Methotrexate bibliography. Vol. I. Lederle Laboratories, New York.
69. Burchenal, J.H., et al. Chemotherapy of leukemia. IV. Effect of folic acid derivatives on transplanted mouse leukemia. Proc. Soc. Exp. Biol. Med. 71, 381(1949).
70. Burchenal, J.H., et al. The effects of the folic acid antagonists and 2,6-diaminopurine on neoplastic disease. Cancer. 4, 549(1951).
71. Skipper, H.E., et al. On the curability of experimental neoplasms. I. Amethopterin and mouse leukemias. Cancer Research. 17, 717(1957).
72. Burchenal, J.H., et al. Prevention of chemotherapeutic effects of 4-amino-N¹⁰-methylpteroylglutamic acid on mouse leukemia by citrovorum factor. Proc. Soc. Exp. Biol. Med. 74, 735(1950).
73. Whiteside, J.A., et al. Intrathecal amethopterin in neurological manifestations of leukemia. A.M.A. Arch. Int. Med. 101, 2(1958).
74. Welch, Arnold D. The problem of drug resistance in cancer chemotherapy. Cancer Research. 19, 359(1959).
75. Novak, E., and Novak, E.R. "Textbook of Gynecology." Williams and Wilkins, Baltimore, 1956.
76. Li, M.C.; Hertz, R.; and Bergenstal, D.M. Therapy of choriocarcinoma and related trophoblastic tumors with folic acid and purine antagonists. New England Jour. Med. 259, 66(1958).

77. Hertz, R., et al. Chemotherapy of chorioncarcinoma and related trophoblastic tumors in women. Jour. Am. Med. Assn. 168, 845(1958).
78. Li, M.C., et al. Effect of Methotrexate therapy upon choriocarcinoma and chorioadenoma. Proc. Soc. Exp. Biol. Med. 93, 361(1956).
79. Li, M.C.; Spencer, D.B.; Hertz, R.; and Lubs, H.A. Suppression of trophoblastic tumors in man by antifolic and antipurine compounds. Proc. Amer. Assoc. Cancer Research. 2, 3, 226abs(March, 1957).
80. Holland, J.F. Methotrexate therapy of metastatic chorioncarcinoma. Am. Jour. Obs. Gyn. 75, 195(1958).
81. Hertz, R., et al. Trophoblast and its tumors. Ann New York Acad. Sci. Vol. 80, Art 1.
82. Nichol, C.A., and Welch, A.E. On the mechanism of action of Aminopterin. Proc. Soc. Exp. Biol. Med. 74, 403(1950).
83. Li, M.C.; Nixon, W.E.; and Freeman, M.V. Increase of urinary citrovorum factor activity in patients receiving methotrexate(amethopterin). Proc. Soc. Exp. Biol. Med. 97, 29(1958).
84. Fountain, J.R., et al. Distribution of amethopterin in normal mouse tissues following intravenous injection. Proc Soc. Exp. Biol. Med. 81, 193(1952).
85. Freeman, M.V. The fluorometric measurement of the absorption, distribution, and excretion of single doses of 4-amino-10-methyl-pteroylglutamic acid(Amethopterin) in man. Jour. Pharm. Exp. Therap. 122, 154(1958).
86. Wollner, N; Murphy, M.A.; and Gordon, C.S. A study of intrathecal Methotrexate. Proc. Am. Assoc. Cancer Research. 3, 1, 74(March, 1959).
87. Freeman, M.V. Occurrence of material detectable by fluorescent measurements in urine following the administration of Amethopterin. Proc. Am. Assoc. Cancer Research. 2, 3, 203abs(March, 1957).
88. Goldin, A., et al. Prolongation of the lifespan of mice with advanced leukemia(L1210) by treatment with halogenated derivatives of amethopterin. Jour. Nat. Cancer. Inst. 22, 811(1959).
89. Goldin, A., and Humphreys, S.R. Studies on immunity in mice surviving systemic leukemia L1210. Jour. Nat. Cancer. Inst. 24, 283(1960).

90. Mayer, K, et al. "Canine Surgery." American Veterinary Publications, Inc. Evanston, Illinois, 1957.
91. Miller, M.E. "Guide to the Dissection of the Dog." Edwards Bros., Inc., Ann Arbor, Mich., 1952.
92. Bratton, A.C., and Marshall, E.K., Jr. A new coupling component for sulfonilamide determination. Jour. Biol. Chem. 128, 537(1939).
93. Freeman, M.V., and Zubrod, C.G. Chemical measurement of amethopterin in plasma and urine. Fed. Proc. 15, 1, 424abs(March, 1956).
94. Freeman, M.V. A fluorometric method for the measurement of 4-amino-10-methylpteroylglutamic acid(amethopterin) in plasma. Jour. Pharmacol. and Exp. Therap. 120, 1(1957).
95. McCoombs, R.P. "Internal Medicine." The Year Book Publishers, Inc. Chicago, Illinois, 1956.

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